BIO-RESEARCH CONSULTANTS, INC.
9 Commercial Avenue
Cambridge, Massachusetts 02141

R E P O R T

to

Report No. 24 (Final)

Contract C-172 (C-193)

Covering the period:

April 16, 1970 through April 30, 1970

Report Prepared by:

Cornelis G. Van Dongen, Ph.D., Research Associate

Distribution:

To Client: Dr. Wakeham - Original
Dr. Osdene - Copy 2
Mr. Carpenter - Copy 3

Date August 16, 1971

This document includes 5 pages, 6 tables and 5 figures.

FINAL REPORT
C-172 (C-193)
from
BIO-RESEARCH CONSULTANTS, INC.
Cambridge, Massachusetts 02141

Bio-Research Consultants, Inc.

F-I-N-A-L R-E-P-O-R-T

The objective of Project C-172 was to develop a medicated cigarette. Medication would be incorporated into the cigarette and administration of this medication to the patient would be through inhalation. During the initial phases of C-172, a number of compounds were considered for the treatment of a variety of clinical problems, and several pilot studies were conducted prior to zeroing in on the specific objective -- to enrich cigarettes with atropine for the purpose of therapeutic use with clinical problems involving abnormally high glandular secretions. The clinical problems could theoretically be present in various areas, such as gastroenterology, sweating, salivation, and even reproduction.

The decision to work toward the development of an atropine-enriched cigarette was made primarily because of the facts that: 1) Atropine has been studied extensively from pharmacological and therapeutic standpoints. 2) Atropine is widely used in clinical practice. 3) Any new means of administration of atropine would inherit a broad range of potential applications. 4) The literature provides some qualitative experimental evidence with humans, suggesting that atropine can be transferred by cigarette whole smoke (Holmstedt, B. and Wallen, O.: Drug Administration by means of Cigarettes. Arch. Int. Pharmacol. 119: 273-293, 1959). This justifies that this experimental approach deserves careful quantitative studies.

Initially, the experiments should definitely not be done with humans. Aside from any legal problems, the cost of experiments with humans for this purpose would be prohibitive. It was therefore decided to do our experimental work with laboratory animals, preferably the least expensive ones, such as the mouse.

With regard to the selection of a physiological process, salivary secretion was chosen above any other of a variety of processes, parasympathetically controlled, because of the fact that it is the most sensitive one. This would allow the monitoring of even very small pharmacological dosages.

The two considerations stated above pointed to the need for an in vivo mouse biological assay which would use the salivary

secretion process. An extensive search of the literature did not reveal the availability of such a method. We therefore developed an in vivo mouse bioassay whereby salivary secretion could be measured quantitatively on large numbers of nontraumatic, unanesthetized mice (a paper on this new methodology was presented at the fall meeting of the American Society for Pharmacology and Experimental Therapeutics in 1969, a copy of which is attached as Appendix No. 1 to this report). In addition to the technical aspects of the method, the paper presents quantitative information on the effects of various physiological conditions, such as sex, strain and individual, on salivary secretion.

Once the "normal" salivary performance of mice was established, we determined to what degree salivary secretion could be modified by chemical treatments. A broader data base of mouse salivation would thus be established for comparison with results in other species. Moreover, studies on the effects of atropine on the salivary secretion of the mouse would serve our specified objective. Appendix No. 2 consists of a paper on this subject which was presented at the annual meeting of the American Society for Pharmacology and Experimental Therapeutics in Atlantic City, N.J. in 1970.

The next logical step in this project appeared to be an extensive study of the effects of cigarette smoke on the salivary secretion of mice, which was carried out. The studies compared the effects of whole smoke vs. gas phases, various cigarette brands, and various smoke concentrations, from the low pharmacological through the high toxicological dose levels. The treatments were frequently tested on, not one, but two, different strains of mice or even on specified subgroups of individuals within a strain. These studies established that the salivary performance of mice in response to low cigarette smoke concentrations can be used as an indicator to estimate quantitatively the acute toxicity of these smokes at higher concentrations. Experimental data from these studies were presented at the annual meeting of the Society for Toxicology in Washington, D.C. (1971), and at the meeting of the American Industrial Hygiene Association in Toronto, Canada (1971). Copies of these papers are attached as Appendices III and IV.

This issue was raised: Are the effects on salivation, in response to exposure by cigarette smokes, due to the nicotine content of the various tobaccos? Our studies with nicotine on salivation led to the conclusion that the smoke effect on salivary performance can well be explained by the pharmacological action of nicotine alone. However, our studies do not exclude the possibility that factors other than

nicotine might also be responsible. Only very carefully conducted quantitative additional studies might elucidate this. The effects of nicotine on salivary performance of two different strains of mice were reported at the FASEB meetings in Chicago (1971) (see Appendix No. V). Our nicotine studies also contributed to a basic understanding of the pharmacology of nicotine. We succeeded to define the required physiological conditions of the animal to obtain reproducible salivary responses with nicotine and we can now explain the controversial results of nicotine on salivation in the literature.

Finally, after Project C-172 had already expired, studies were conducted on our own to examine the effects of exposures to atropine-enriched cigarettes on the salivary performance of mice. Male C57BL/6 J mice (approximately 30 weeks old), which had been caged for some time while wearing collars, were used. These animals had been adapted to the salivation procedure and were all in good health. Their salivary performance was determined according to standardized procedures four times, with a one-hour interval between measurements. The first and second measurements established the control salivary performance of individual mice during that particular day. A ten-minute smoke exposure was applied immediately prior to the third measurement. The fourth measurement was performed to examine any possible aftereffect to the treatments. During the time interval between any two salivation measurements, the mice were returned to their "treatment" cages, which were supplied with adequate food and water.

The experimental cigarettes were Pall Mall, cut to 65 mm length. By means of a syringe with long needle, the cigarettes were homogeneously impregnated with 1 ml of absolute alcohol containing either 0, 0.4, 4.0 or 40 mg of atropine. The plain cigarettes and the four alcohol-treated groups of cigarettes were subsequently dried for one-half hour in a 40° C oven and then placed in a desiccator for approximately 18 hours, after which they were taken out for immediate use in the smoking machine.

The conditions of the smoking machine were standardized to 35 ml puffs of two seconds' duration, taken at intervals of 58 seconds. The smoke persisted for 15 seconds in the inhalation chamber and was expulsed in three seconds, followed by a 40-second fresh air purge. Ten puffs were used in each case, the first five puffs of each of two sets of cigarettes being smoked without interruption in the one-puff-per-minute cycle. In order to obtain 10% smoke concentrations, we burned one experimental cigarette at a time.

A single experiment started off with determination of the first and second salivation measurements on 72 mice. The sum of these two measurements served to rank order the mice according to salivary performance and allowed for any unexpected loss of mice. Next, 36 mice were assigned systematically to any one of six treatment groups (no exposure, exposure to whole smoke of Pall Mall, and exposure to the various atropine-enriched Pall Mall cigarettes). The remaining mice were eliminated from the experiment. Thus, the third and fourth salivation measurements were limited to a maximum of 36 mice (six mice in each of six treatment groups). The experiment was repeated once and the results of the two experiments were pooled into a set. A set of data was thus based on four measurements from 72 mice or, maximally, 12 mice per treatment group. One treatment, "machine exposure only," was not included, although it would have made the experiment more complete. The reasons were that it is very difficult to handle simultaneously seven treatment groups, and that earlier experiments had shown repeatedly that no significant differences existed between control and machine exposure when trained mice were used. When analyzing results, the 12 mice were separated into "high" or "low" salivators on the basis of their first and second salivation measurements. Using the Wang computing capability, we obtained significant data by means of established statistical techniques.

Results and Discussion

Tables No. 171 through 176 present the effects of the various treatments on salivary performance of C57BL mice (see also figures 35 through 39). There was dramatic salivary inhibition with any of the exposures of whole cigarette smoke. In addition, salivary inhibition by whole smoke was higher with the atropine-enriched cigarettes relative to that of their controls. Finally, it was shown that salivary inhibition is related to the atropine dose in a dose-response fashion. If substantiated by chemical experimental evidence, these results would then prove that the administration of atropine by means of inhalation is technically possible.

Conclusions: I. Strong experimental evidence is presented for the assumption that atropine is transferred by cigarette whole smokes.
II. The above-described experimental results indicate that the objectives of C-172 have been achieved.

Potential Practical Applications for Mouse Salivation Bioassay:

1. Biological characterization of various cigarette brands;

2. Prediction of acute toxicity in cigarette smoke;

3. Testing of efficiency of various cigarette filters;

4. Estimation of degree of biological harmfulness of the particulate phase in whole cigarette smoke (still to be worked out);

5. Testing of the pleasurability of various additives to cigarettes (still to be worked out).

Table 171

Mean Salivary Performances of Male C57BL Mice When Exposed to 10% Whole Smokes from Atropine-Enriched Pall Mall Cigarettes Immediately prior to the Third Salivation Measurement (Expressed in mm of boundary displacement)

Treatment	No. of Animals	Measurements			
		1	2	3	4
Control	11	56.6	69.0	64.4	46.2
Pall Mall plain	12	64.0	71.8	30.8	40.5
" " ± alcohol	11	64.7	69.7	20.4	24.1
" " + 0.4 mg atropine	12	64.9	69.4	10.7	36.1
" " + 4.0 mg atropine	12	73.8	64.3	6.3	23.5
" " + 40.0 mg atropine	12	64.3	70.1	1.5	22.3

Table 172

ANALYSIS OF VARIANCE
(Nonselected C57BL/6 J mice)

Measurement 1

Source	DF	SS	MS	F cal	F 95%	F 99%
Treatments	5	1,723	345	0.39	2.37	3.34
Error	64	55,962	874			
Total	69	57,685				

Measurement 2

Source	DF	SS	MS	F cal	F 95%	F 99%
Treatments	5	380	76	0.07	2.37	3.34
Error	64	72,030	1,125			
Total	69	72,410				

Measurement 3

Source	DF	SS	MS	F cal	F 95%	F 99%
Treatments	5	30,264	6,053	20.50**	2.37	3.34
Error	64	18,869	295			
Total	69	49,133				

** Significant at the 1% level of probability

Measurement 4

Source	DF	SS	MS	F cal	F 95%	F 99%
Treatments	5	5,956	1,191	1.98	2.37	3.34
Error	64	38,614	603			
Total	69	44,570				

Table 173

Mean Salivary Performances of High-salivating Male C57BL Mice When Exposed to 10% Whole Smokes from Atropine-enriched Pall Mall Cigarettes Immediately Prior to the Third Salivation Measurement (Expressed in mm of boundary displacement)

Treatment	No. of Animals	Measurements			
		1	2	3	4
Control	5	85	86	67	48
Pall Mall, plain	6	85	105	31	48
Pall Mall + alcohol	5	83	97	27	23
Pall Mall + 0.4 mg atropine	6	87	86	8	27
Pall Mall + 4.0 mg atropine	6	95	83	5	30
Pall Mall + 40.0 mg atropine	6	77	98	1	22

Table 174

ANALYSIS OF VARIANCE
(High-salivating C57BL/6 J mice)

Measurement 1

Source	DF	SS	MS	F cal	F 95%	F 99%
Treatment	5	1,118	224	0.57	2.56	3.75
Error	28	10,943	391			
Total	33	12,061				

Measurement 2

Source	DF	SS	MS	F cal	F 95%	F 99%
Treatment	5	2,207	441	0.62	2.56	3.75
Error	28	19,895	711			
Total	33	22,102				

Measurement 3

Source	DF	SS	MS	F cal	F 95%	F 99%
Treatment	5	16,242	3,248	10.60**	2.56	3.75
Error	28	8,579	306			
Total	33	24,821				

** Significant at the 1% level of probability

Measurement 4

Source	DF	SS	MS	F cal	F 95%	F 99%
Treatment	5	3,983	797	1.25	2.56	3.75
Error	28	17,919	640			
Total	33	21,902				

Table 175

Mean Salivary Performances of Low-salivating Male C57BL Mice When Exposed to 10% Whole Smokes from Atropine-enriched Pall Mall Cigarettes Immediately Prior to the Third Salivation Measurement (Expressed in mm of boundary displacement)

Treatment	No. of Animals	Measurements			
		1	2	3	4
Control	5	24	50	58	48
Pall Mall, plain	6	43	39	31	33
Pall Mall + alcohol	6	50	47	15	25
Pall Mall + 0.4 mg atropine	6	43	53	14	46
Pall Mall + 4.0 mg atropine	6	52	46	8	18
Pall Mall + 40.0 mg atropine	6	52	42	2	23

Table 176

ANALYSIS OF VARIANCE
(Low-salivating C57BL/6 J mice)

Measurement 1

Source	DF	SS	MS	F cal	F 95%	F 99%
Treatment	5	3,039	608	1.27	2.55	3.73
Error	29	13,931	480			
Total	34	16,970				

Measurement 2

Source	DF	SS	MS	F cal	F 95%	F 99%
Treatment	5	758	152	0.36	2.55	3.73
Error	29	12,151	419			
Total	34	12,909				

Measurement 3

Source	DF	SS	MS	F cal	F 95%	F 99%
Treatment	5	11,285	2,257	7.10**	2.55	3.73
Error	29	9,178	316			
Total	34	20,463				

** Significant at the 1% level of probability

Measurement 4

Source	DF	SS	MS	F cal	F 95%	F 99%
Treatment	5	4,403	881	1.40	2.55	3.73
Error	29	18,245	629			
Total	34	22,648				

Figure 35

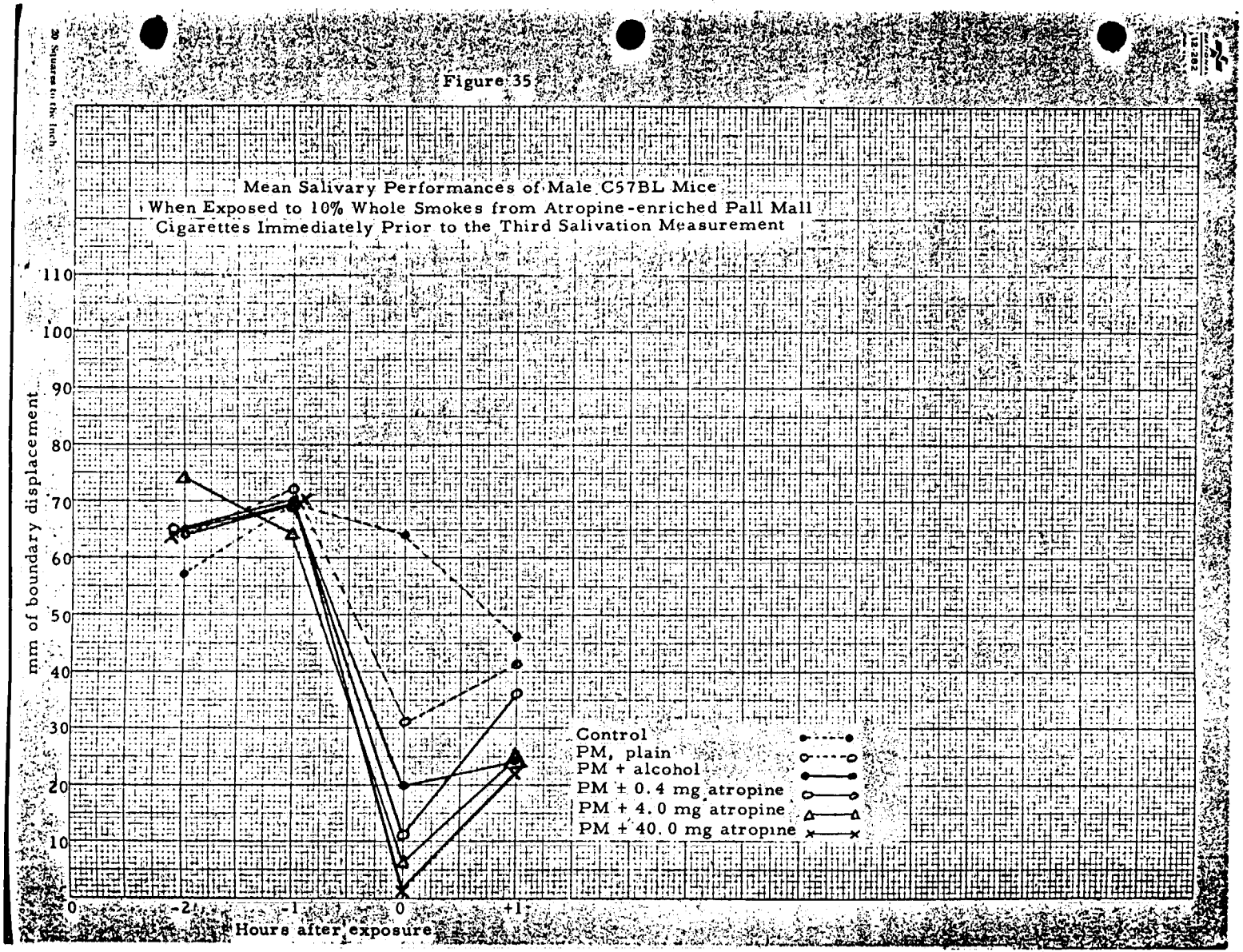

Figure 36

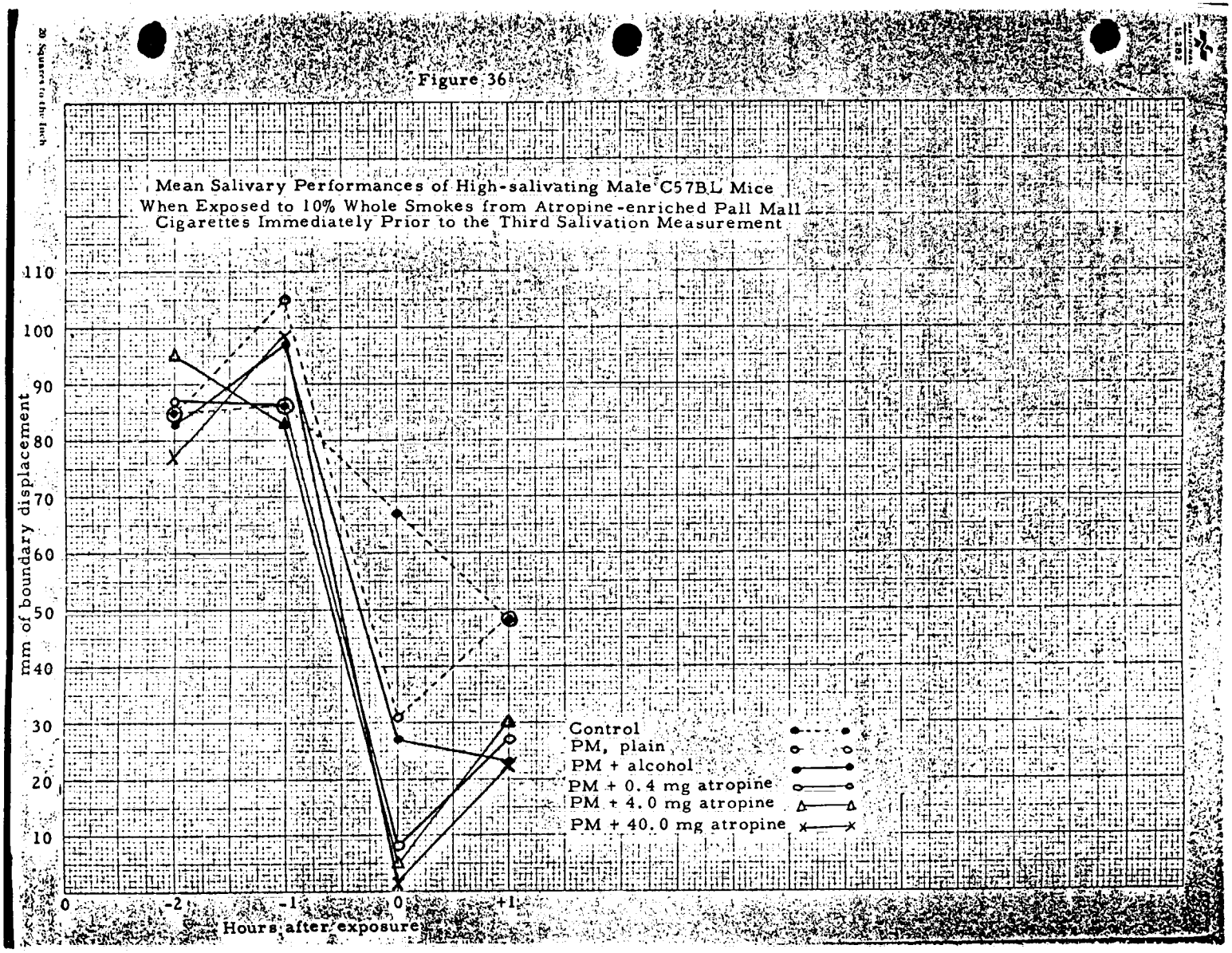

Figure 37

Mean Salivary Performances of Low-salivating Male C57BL Mice When Exposed to 10% Whole Smokes from Atropine-enriched Pall Mall Cigarettes Immediately Prior to the Third Salivation Measurement

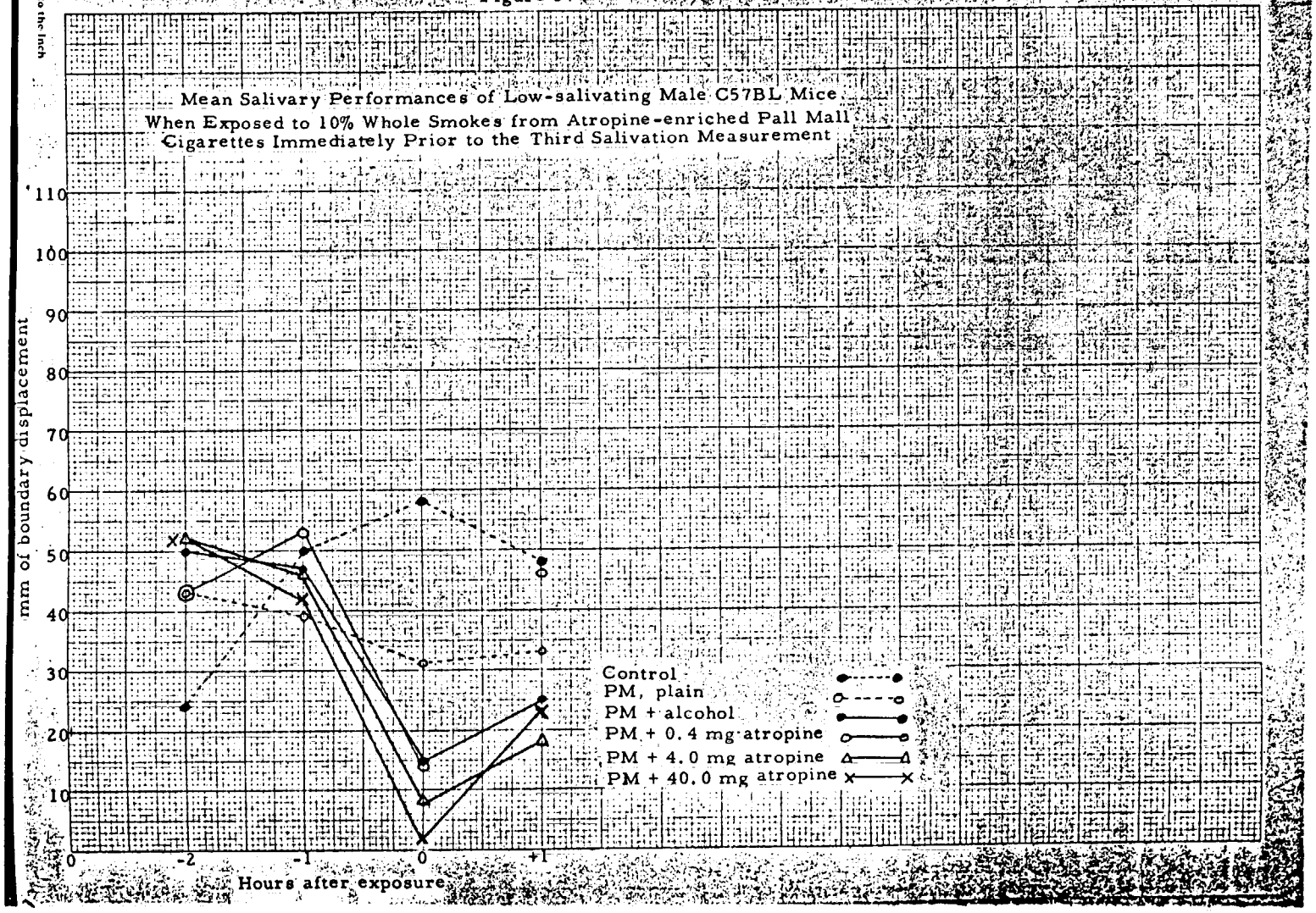

Figure 38

EFFECT OF WHOLE SMOKES FROM PALL MALL (PM) CIGARETTES ENRICHED WITH VARYING DOSES OF ATROPINE (AT) ON SALIVARY PERFORMANCE OF POOLED, HIGH- AND LOW-SALIVATING MALE C57BL/6J MICE (MEASURED IMMEDIATELY AFTER EXPOSURE)

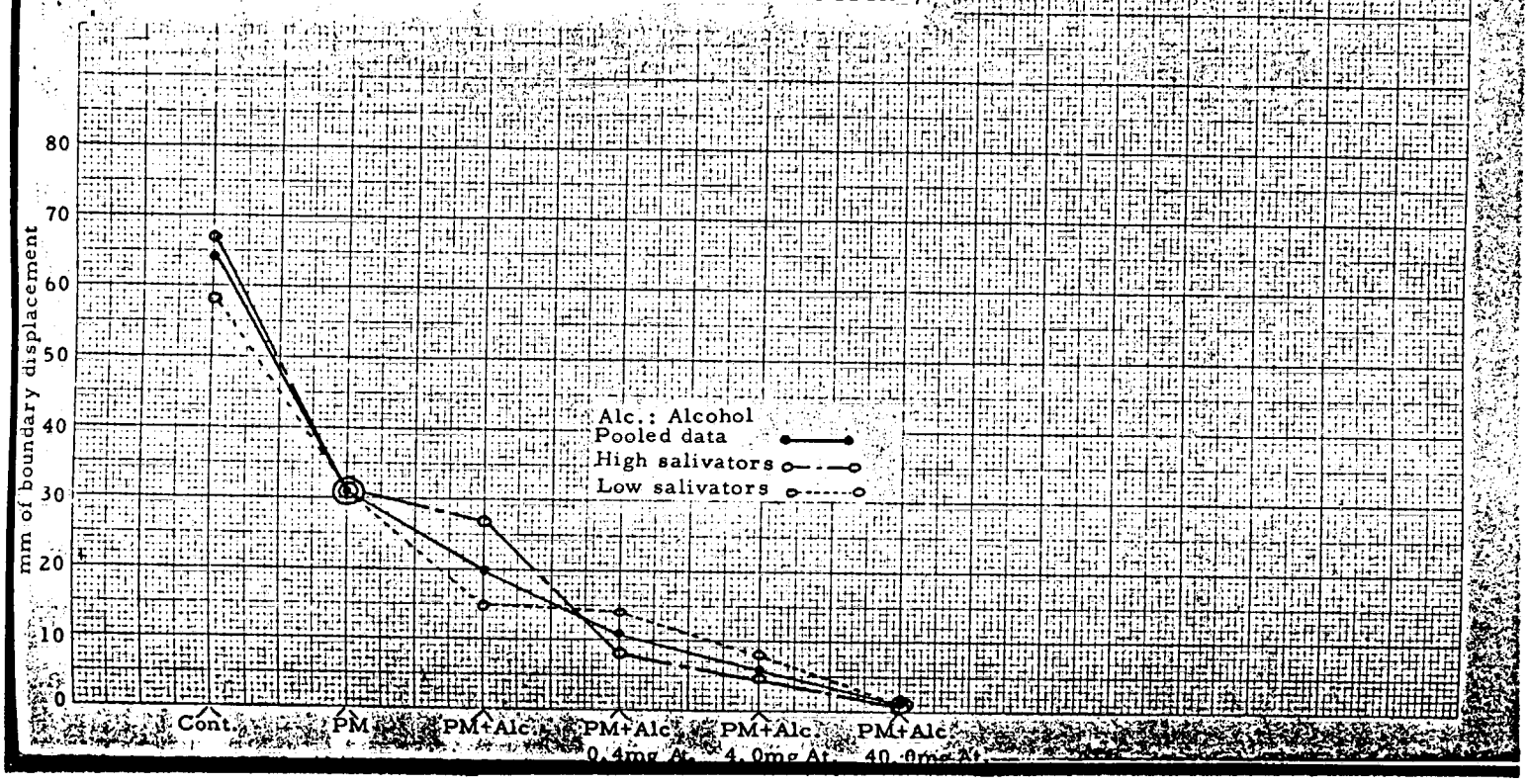

Figure 39

EFFECT OF WHOLE SMOKES FROM PALL MALL (PM) CIGARETTES ENRICHED WITH VARYING DOSES OF ATROPINE (AT) ON SALIVARY PERFORMANCE OF POOLED, HIGH- AND LOW-SALIVATING MALE C57BL/6J MICE (MEASURED ONE HOUR AFTER EXPOSURE)